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Sting frequency and progeny production of lab-cultured *Cotesia marginiventris*

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Abstract A laboratory experiment was conducted to determine the impact of sting frequency on progeny production of *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae), a solitary, koinobiont endoparasitoid of noctuid pests. In replicated trials, young, mated, host-deprived *C. marginiventris* females were exposed to three *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) early instar larvae, one at a time, in Petri dish arenas, and observed as they stung these hosts once, twice, or thrice. The average amount of time that elapsed between stinging a host ranged from a minimum of 3 min, when females were exposed to unstung hosts, to a maximum of 8–11 min when females were exposed to previously stung hosts. Sting frequency had no effect on the yield of *C. marginiventris* progeny; approximately 90% of hosts, in all treatment groups, yielded a parasitoid mature larva, which spun a cocoon. No hosts yielded more than one parasitoid. The percentage of adult female progeny tended to increase with sting frequency; hosts that were stung thrice yielded 60% female *C. marginiventris* adults. The production of female progeny may depend on the egg allocation pattern of *C. marginiventris* females as well as the competitive interactions between siblings developing inside a shared host. This study suggests that *S. exigua* larvae can sustain 2–3 stings without diminishing their ability to yield a *C. marginiventris* mature larva. Provisioning enclosures with enough hosts to exceed the daily (per capita) oviposition rate of *C. marginiventris* females might limit the repeated stinging of the same hosts.

Keywords Endoparasitoid · Rearing · Sex ratio · Solitary · Self-superparasitism

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Introduction

Cotesia marginiventris (Cresson) (Hymenoptera: Braconidae) is a solitary, arrhenotokous, koinobiont endoparasitoid of pestiferous noctuids (Loke et al. 1983; Novoa and Luna 1996) and has been considered for augmentative biological control of these caterpillars on vegetables grown in greenhouses (Urbaneja et al. 2002). A prerequisite to augmentative (i.e., inundative) releases is the production of large quantities of quality insects (Nordlund 1998). Refinement and improvement of in vivo methods of rearing *C. marginiventris* are needed, especially since in vitro methods have not been fully-developed. Knowledge of the host-stinging behavior of *C. marginiventris* may contribute to the development of methodologies for producing quality parasitoids. Observing stinging behavior could provide clues to help predict whether this insect has a proclivity to superparasitize hosts in culture systems.

The propensity of insect parasitoids to discriminate between parasitized and unparasitized hosts is well documented (van Lenteren 1981). Despite this ability, hosts that are parasitized more than once are often discovered (van Alphen and Visser 1990; Speirs et al. 1991). In solitary endoparasitoids, superparasitism can occur when a female deposits two or more eggs in a host during two or more distinct attacks. Because offspring must compete for resources in the same host, in all but rare instances, only one larval parasitoid will complete its development, pupate and metamorphose into a healthy adult (Salt 1934). If only one female of a species is involved, the behavior is termed self-superparasitism; when two or more females are involved, it is called conspecific superparasitism (van Dijken and Waage 1987). Self-superparasitism in solitary parasitoids may result in a waste of time and eggs. However, self-superparasitism may be advantageous if the presence of two or more eggs per host increases the overall chance that one parasitoid will survive (van Alphen and Visser 1990), if the encounter rate with healthy (i.e., unparasitized) hosts is low (Hubbard et al. 1999), and if there is fierce intraspecific competition for hosts (Pexton and Mayhew 2005). Conspecific superparasitism can be adaptive if the offspring of the second female to attack the host has a probability of greater than zero of surviving to the adult stage (van Alphen and Visser 1990). Superparasitism may result in an alteration of the sex ratio of adult progeny. It may increase the proportion of females (Darrouzet et al. 2003), males (Li et al. 2001) or have no noticeable effect (van Dijken et al. 1993) on sex ratio of adult progeny.

In some in vivo rearing systems, ovipositing parasitoids are confined to the same enclosure with hosts for up to 24 h (e.g., Ramadan 2004; Riddick 2004). In these enclosures, parasitoids might encounter previously stung (i.e., parasitized) hosts. The number of times a host is stung (i.e., sting frequency) may or may not affect the ability of the host to yield parasitoid progeny. In this study, the stinging behavior of lab-cultured *C. marginiventris* females was noted and the influence of sting frequency on progeny production was determined.

Materials and methods

Insect colonies

Cotesia marginiventris was reared at the USDA-ARS, Biological Control and Mass Rearing Research Unit (BCMRRU), Mississippi State, MS for more than 150 continuous generations. The original parasitoids were from a colony maintained at an USDA-ARS facility in Tifton, Georgia, USA. The culture at BCMRRU was maintained by exposing late 1st to 2nd instar beet armyworms, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), reared on a soybean flour-wheat germ meridic diet (after King and Hartley 1985), to recently-emerged (i.e., 1–2 days old) *C. marginiventris* females for 24 h in a 'sting' box (45.7 × 66.0 × 8.9 cm, W × L × H; 18.9 L clear plastic) provisioned with pure honey and sterile water, in cotton pads. Parasitized larvae were placed within polystyrene rearing trays (with meridic diet) and held in an environmental room (27–28°C, 60–70% RH, and 16 h photophase). After 2 weeks, *C. marginiventris* cocoons (i.e., pupae) were harvested from trays. Adults emerged from cocoons within 5 days, males typically one day before females. Mating can occur within minutes after females emerge (Boling and Pitre 1970). Females held an average of 149 mature eggs in ovaries and lateral oviducts just after emergence (Riddick 2006). Adult females usually live for 2 weeks at rearing temperatures in the laboratory (Riddick 2001) and most female progeny are generated during the first week, when maternal females are exposed to an abundance of host, *S. exigua*, larvae (Tillman 2001).

Experimental protocol

In order to manipulate sting frequency, young (1–2 days old, presumably mated, and host-deprived) *C. marginiventris* females were removed at random from an emergence cage of mixed sexes and placed, individually, within a clean, plastic Petri dish (20 mm depth, 100 mm diam) with a series of *S. exigua* late 1st to early 2nd instar larvae and observed while stinging these hosts once, twice or thrice. In other words, each host was stung 1–3 times by the same individual parasitoid within a replicate trial. Within 1–2 s after being stung, each host regained full mobility. The time (in minutes) that elapsed between stinging of each host was recorded. A schematic flow diagram of the sequence of hosts and the time categories between stinging these hosts is illustrated in Fig. 1A. In this figure host larvae 1, 2, and 3 (H1, H2, and H3) were stung once (.1), twice (.2), or thrice (.3) by the same *C. marginiventris* female. A sting event usually lasted for 1–2 s; afterwards, the female often dragged her ovipositor across the base of the Petri dish, for an additional 1–2 s. Parasitoids never fed on secretions exuding from the site of puncture in the integument of their hosts.

Stung hosts were placed inside separate 50 ml plastic centrifuge tubes containing approximately 20 ml of meridic diet. Each tube was labeled according to the sting frequency that each host larva had been subjected to in the Petri dish arena. A 2-cm diameter hole was bored into the cap of each tube and covered with nylon mesh to provide air circulation. This protocol was initially replicated 10 times on the same day, for a total utilization of 10 *C. marginiventris* females and 30 *S. exigua* larvae. But, the replication was reduced to include only females that eventually produced at least one female progeny (i.e., had been assured of mating prior to the experiment). Therefore, the replication (per day) was reduced to 5 mated females and 15 host larvae. Parasitized larvae (inside tubes) were placed inside a growth chamber (at 26.5°C, 60% RH, and 16 h photophase) and undisturbed until 3rd instar larvae exited their hosts and spun silken cocoons on the inner wall of the tubes. This experiment was replicated 3 times (on 19 November 2001, 14 January 2002, and 11 February 2002).

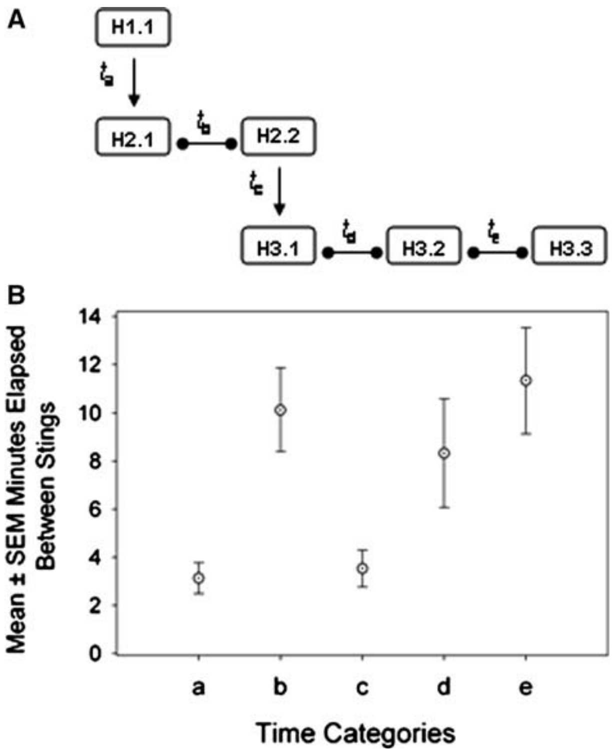

Fig. 1 Schematic flow diagram of host larvae 1, 2, and 3 (H1, H2, H3) stung once (.1), twice (.2), or thrice (.3) by the same *C. marginiventris* female (**A**), and the mean ± SEM number of minutes that elapsed between stings (**B**). Time categories (t_a, t_b, t_c, t_d, t_e) designate minutes that elapsed between consecutive sting events. N = 75 observations. A sample of 5 mated *C. marginiventris* females and 15 *S. exigua* larvae were involved in each trial. These data were representative of three replicate trials

Stung hosts were considered as experimental units. The percentage of host larvae successfully yielding 3rd instar parasitoid larvae and the percentage of parasitoid females emerging from cocoons were determined in relation to sting frequency.

Statistical analysis

Data were analyzed following a completely randomized design and trials served as an additional source of replication. An analysis of variance (ANOVA) was used to test for significant differences in the time that elapsed before mated females stung their hosts once, twice or thrice. An ANOVA also tested the significance of sting frequency on the percentage of hosts yielding parasitoids and the percentage of female progeny emerging from cocoons. Absolute data were log-transformed (using the natural logarithm) and percentage data were arcsine-transformed prior to analysis. Means were considered significantly different when $P < 0.05$; the Tukey's test was used for separation of means after a significant ANOVA. Statistical analyses were performed with SigmaStat (2004). Only untransformed data are presented.

Results and discussion

Each *Cotesia marginiventris* female walked rapidly, examining the base of the Petri dish arena with the antennae. Once initial contact with the first host larva was made, the female decreased its walk speed and vigorously examined the host with its antennae for approximately 1 s. Host quality may have been assessed at this time. Usually, the female walked several centimeters away from the host, but later returned to investigate it one or more times before mounting and then stinging it. The female usually inserted its ovipositor into the dorso-lateral midsection of the *S. exigua* larva. The approximate time to sting a host was only 1–2 s.

The average amount of time that elapsed between stinging a host ranged from a minimum of 3 min, when females were exposed to unstung hosts, to a maximum of 8–11 min when females were exposed to previously stung hosts (Fig. 1B). The time that elapsed before females decided to sting a host larva differed significantly between time categories ($F = 8.2$; df = 4, 70; $P < 0.001$). More time elapsed before females stung the second host (H 2) for the second time than the first time (t_b vs t_a, respectively; Fig. 1A, B). More time elapsed before females stung H 2 the second time than to sting the third host (H 3) for the first time (t_b vs t_c, respectively). Also, more time elapsed before females stung H 3 the third time than the first time (t_e vs t_c, respectively) or H 2 the first time (t_e vs t_a, respectively). The tendency to require more time to re-sting hosts under no-choice conditions in this study suggests that *C. marginiventris* females have some ability to discriminate between self-parasitized and unparasitized hosts.

Host-stinging behavior of *C. marginiventris* could involve the insertion of the ovipositor into the host to (1) assess host quality (i.e., determine if it had already been parasitized), (2) deposit an egg and associated fluids into the host, or (3) place a chemical marker inside the host after oviposition is complete. Observations of host-stinging behavior did not disclose the relative frequency of the three components. The time involved in ovipositor insertion into and removal from stung hosts in this study did not appear to differ between the first, second or third sting. Also, body movements of females in the process of stinging were rather consistent; thus, not providing any clues as to whether an egg had been inserted, and if so, whether it had been fertilized. Without dissection, it is not possible to state with certainty that the second and third hosts always contained 2 and 3 parasitoid eggs, respectively. However, host-stinging to lay an egg (rather than to assess host quality) probably occurs most of the time a female decides to attack a host that it has already stung, recently.

The time interval between stings (i.e., apparent ovipositions) increased significantly as females of another solitary braconid, *Dinocampus coccinellae* (Schrank), stung the same coccinellid host, *Coccinella septempunctata* L., once, twice, or thrice in Petri dish arenas in the laboratory (Okuda and Ceryniger 2000). The authors concluded that *D. coccinellae* females were capable of discriminating between self-parasitized and unparasitized host beetles.

Sting frequency had no effect on the yield of *C. marginiventris* progeny from host larvae ($F = 0.18$; df = 2, 6; $P = 0.84$); hosts that had been stung twice or thrice were not more successful at yielding a parasitoid progeny than those that were stung just once (see Table 1). In all cases, a single *C. marginiventris* mature larva emerged from a given host. The observation that 87% of the hosts, on average, stung just once

Table 1 Influence of sting frequency on the mean ± SEM percentage of host (*S. exigua*) larvae yielding parasitoid (*C. marginiventris*) mature larvae and the mean ± SEM percentage of adult female progeny

Sting freq.	Hosts yielding *Cotesia*	Emerging adults	Female progeny
1	86.7 ± 13.3 a	100	13.3 ± 6.7a
2	93.3 ± 6.7 a	100	33.3 ± 6.7 a b
3	86.7 ± 6.7 a	100	60.0 ± 0.0 b

Mean values followed by a different letter, in a column, are significantly different ($P < 0.05$; Tukey's test). Each of three trials consisted of 15 *S. exigua* 1st–2nd instar larvae, 5 per treatment group. Five previously-mated *C. marginiventris* females were in each trial; each female stung three host larvae once, twice, or thrice, in sequence

yielded a parasitoid mature larva suggests that females usually parasitize the first host encountered. That an average of 13% of hosts stung once did not yield any progeny could be a reflection of the frequency at which stinging behavior did not result in deposition of an egg. Note that approximately 4.6% of initial stings by the solitary braconid *Microplitis mediator* (Haliday) resulted in no egg being laid into its noctuid host, *Pseudeletia separata* Walker, in the laboratory (Qin et al. 1999).

When stinging did in fact result in oviposition, only one *C. marginiventris* progeny was capable of completing its development from resources provided in one host larva in this study. Consequently, a number of eggs deposited in hosts that were stung two or three times were wasted. Egg wastage may or may not reduce, significantly, the net fecundity of *C. marginiventris* because females emerge with more than enough mature eggs, ready to oviposit into hosts, and honey-fed females can mature many more eggs in several days in the laboratory (Riddick 2007).

Successful parasitoid progeny emerged from hosts and proceeded to spin a cocoon, individually, near the remains of their hosts in all treatment groups. Within approximately 5 days, mature larvae metamorphosed into pupae, then into adults that emerged from cocoons. Although emerged adults seemed healthy (i.e., mobile and fully-formed), their body size and weight were not recorded.

Sting frequency had a significant effect on the percentage of female progeny emerging from cocoons. A greater percentage of female progeny resulted from hosts that were stung thrice than once ($F = 8.5$; df = 2, 6; $P = 0.02$; Table 1). If an egg was inserted into a host at least 87% of the time, host 1 would contain parasitoid egg no. 1; host 2, egg nos. 2 and 3; and host 3, egg nos. 4, 5, and 6. If sting frequency was a direct reflection of oviposition rate, an adult female was generated 60% of the time when parasitoid egg nos. 4, 5, and 6 were present in a shared (i.e., self-superparasitized) host. This represents a slightly female-biased sex ratio of adult progeny.

The sex ratio of *C. marginiventris* ranged from slightly female-biased (Tillman 2001), unbiased (Boling and Pitre 1970), to slightly male-biased (Kunnalaca and Mueller 1979) in the laboratory. Note that a purportedly high level of self-superparasitism generated an unbiased (1: 1.02) or male-biased (1: 0.64) sex ratio when one or two progeny, respectively, emerged from the same *S. exigua* larva (Riddick 2002). Only one *C. marginiventris* emerged from a *S. exigua* larva in this study.

One possible mechanism to explain the tendency of female progeny to increase along with sting frequency is that inexperienced females might lay a male egg in the first host and then lay progressively more female eggs in available hosts, regardless of host status (i.e., unparasitized vs parasitized). This pattern could continue until the sex ratio is near the range that typifies this species (i.e., ~45–55% adult females). Waage (1986) indicated that some endoparasitoids allocate unfertilized (i.e., male) eggs to hosts at the start of the egg-laying sequence and the laying of female eggs increases gradually soon thereafter.

Another possibility is that female larvae could out-compete male siblings that are developing in the same host. First instar larvae of a mymarid, *Anaphes victus* Huber, vie for limited resources in a shared host and males experience higher mortality (van Baaren et al. 1999). Self-superparasitism altered the sex ratio of adult progeny of an ichneumonid, *Diadegma semiclausum* Hellén, due to differential mortality of male larvae inside hosts (Yang et al. 1994). Although *C. marginiventris* 1st instar larvae possess sickle-shaped mandibles (Boling and Pitre 1970) and are capable of killing siblings (Riddick 2002), there is no solid evidence of differential mortality of developing *C. marginiventris* larvae. Morphological characters to distinguish male from female *C. marginiventris* larvae are not known.

In conclusion, the production of female progeny may depend on the egg allocation pattern of *C. marginiventris* females as well as the competitive interactions between siblings developing inside a shared host. This study suggests that *S. exigua* larvae can sustain 2–3 stings without diminishing their ability to yield a *C. marginiventris* mature larva. Thus, a low level of ''putative'' self-superparasitism is not detrimental. Provisioning enclosures with enough hosts to exceed the daily, per capita, oviposition rate of *C. marginiventris* females might limit the repeated stinging of the same hosts.

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